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RESEARCH ARTICLE

Intraspecific comparison of population structure, genetic diversity, and dispersal among three subspecies of Townsend's big-eared bats, *Corynorhinus townsendii townsendii, C. t. pallescens*, and the endangered *C. t. virginianus*

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Abstract Townsend's big-eared bat, *Corynorhinus townsendii*, is distributed broadly across western North America and in two isolated, endangered populations in central and eastern United States. There are five subspecies of *C. townsendii*; *C. t. pallescens*, *C. t. australis*, *C. t. townsendii*, *C. t. ingens*, and *C. t. virginianus* with varying degrees of concern over the conservation status of each. The aim of this study was to use mitochondrial and microsatellite DNA data to examine genetic diversity, population differentiation, and dispersal of three *C. townsendii* subspecies. *C. t. virginianus* is found in isolated populations in the eastern United States and was listed as endangered under the Endangered Species Act in 1979. Concern also exists about declining populations of two western subspecies, *C. t. pallescens* and *C. t. townsendii*. Using a comparative approach, estimates of the genetic diversity within populations of the endangered subspecies, *C. t. virginianus*, were found to be significantly lower than within populations of the two western subspecies. Further, both classes of molecular markers revealed significant differentiation among regional populations of *C. t. virginianus* with most genetic diversity distributed among populations. Genetic diversity was not significantly different between *C. t. townsendii* and *C. t. pallescens*. Some populations of *C. t. townsendii* are not genetically differentiated from populations of *C. t. pallescens* in areas of sympatry. For the western subspecies gene flow appears to occur primarily through male dispersal. Finally, geographic regions representing significantly differentiated and genetically unique populations of *C. townsendii virginianus* are recognized as distinct evolutionary significant units.

Keywords *Corynorhinus townsendii virginianus* · Mitochondrial DNA · Microsatellite DNA · Endangered species · Genetic diversity

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Introduction

Concern over apparent decline and continuing threats to populations of North American big-eared bats, *Corynorhinus townsendii*, make it critical to ascertain the precise status of populations in order to develop appropriate management and conservation strategies. However, bats in general are difficult to study (Burland and Wilmer 2001), and *C. townsendii*, in particular, is quite elusive, which has made it difficult to attain accurate population information through traditional ecological studies. *Corynorhinus townsendii* is a medium-sized (10–12 g) North American bat belonging to the family Vespertilionidae and the tribe Plecotini, which is ascribed five subspecies (Handley 1959; Piaggio and Perkins 2005). *C. townsendii* populations have been found from sea level to 3,188m (Pearson et al. 1952; Szewczak et al. 1998; Pierson et al. 1999) but appear to be limited by roosting habitats, which are primarily

underground features such as caves or abandoned mines. This species has been listed as vulnerable to extinction (VU) by the World Conservation Union's 2004 IUCN Red List of threatened species (www.redlist.org), yet little is known about population dynamics or genetic diversity of these bats (Humphrey and Kunz 1976; Pearson et al. 1952; Weyandt et al. 2005). The individual subspecies of *C. townsendii* have been a focus of considerable conservation concern. There are two subspecies that occupy isolated and disjunct distributions, *C. t. virginianus* and *C. t. ingens* (Fig. 1), and both are listed as federally endangered under the Endangered Species Act (USFWS 1979). The two western subspecies, *C. t. pallescens* and *C. t. townsendii*, have been candidates for threatened or endangered status (USFWS 1979, 1989, 1994) when that designation existed. Today, these two subspecies are listed in all western states and British Columbia as either vulnerable, Species of Concern, or Sensitive Species by the western regions of the U.S Forest Service and the Bureau of Land Management (Pierson et al. 1999; Western Bat Working Group 1998).

Corynorhinus townsendii virginianus and *C. t. ingens*, the subspecies listed as endangered (USFWS 1979), roost mainly in caves, although *C. t. virginianus* sometimes uses abandoned coal and hard rock mines. Their declines have been attributed to a sharp increase in cave recreation that has occurred since the late 1950's and to an intolerance of these bats to human disturbance (Humphrey and Kunz 1976). Further, these subspecies live in disjunct regions with little or no possibility for gene flow. In the western United States, *C. t. townsendii* and *C. t. pallescens*

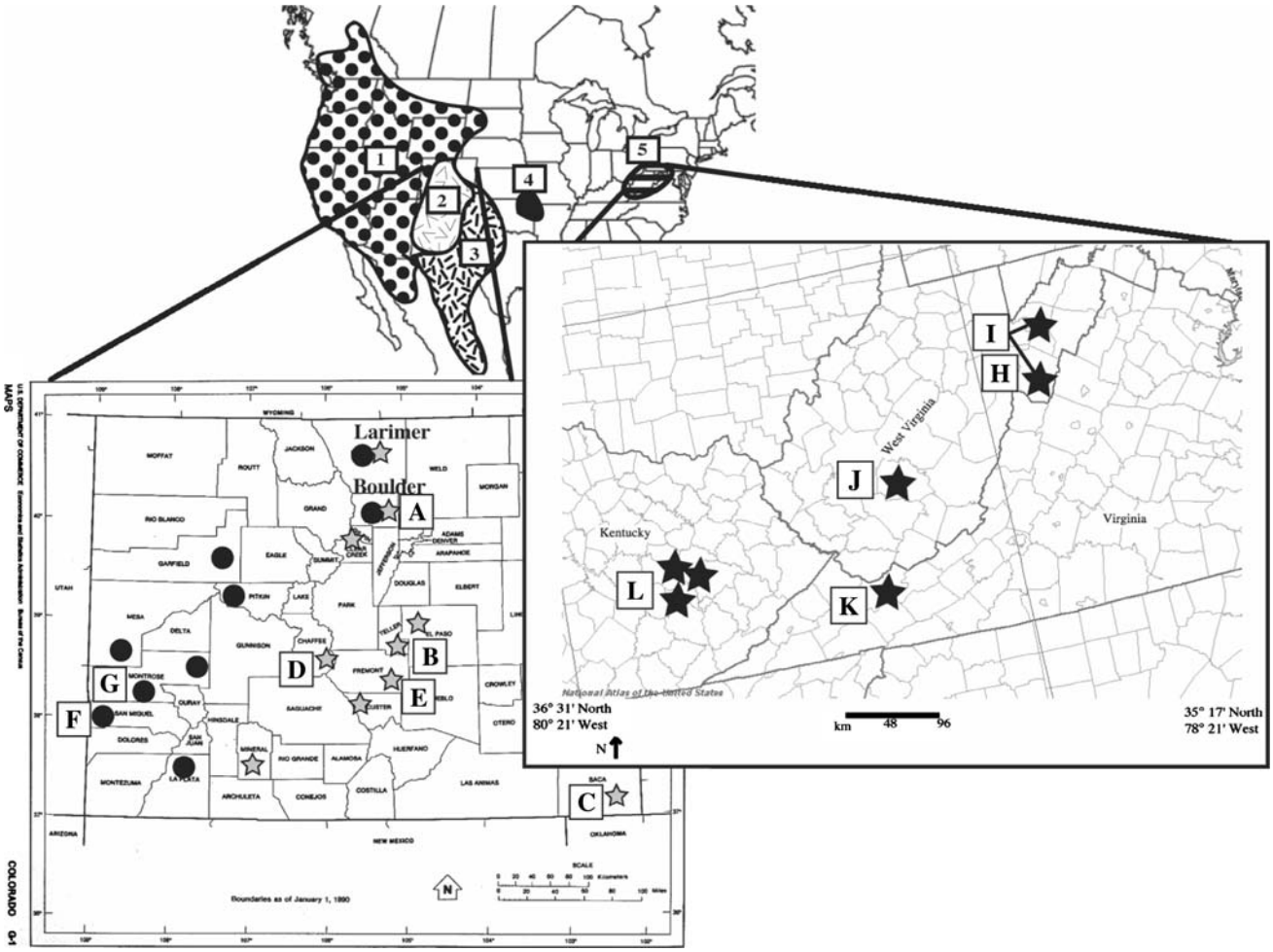

Fig. 1 Distribution of *C. townsendii* with ranges of subspecies. Subspecies are labeled as follows: (1) *C. t. townsendii*, (2) *C. t. pallescens*, (3) *C. t. australis*, (4) *C. t. ingens*, (5) *C. t. virginianus* (Piaggio and Perkins 2005). Areas of microgeographic examinations are shown in detail with areas sampled (black circles = *C. t. townsendii*, gray stars = *C. t. pallescens*, and black stars = *C. t. virginianus*) marked and populations labeled. Populations correspond to, *C. t. pallescens* (**a**–**e**), *C. t. townsendii* (**f**, **g**), and *C. t. virginianus* (**h**–**l**). Black lines from population I signify that this population is made up of individuals from caves in both Pendleton County and Grant County. Population H is a single cave also found in Pendleton County, West Virginia. However, all individuals in population I are 32.2 km away from population H. (Map from the University of Texas, Austin, Perry Casteñeda Library Map Collection on-line http://www.lib.utexas.edu/maps/)

populations are primarily found roosting in abandoned mines, although there are records of roosts in caves and abandoned structures (Kunz and Martin 1982). Large dead trees that may have served as roosts historically have been cleared and in recent times these bats have taken up residence in abandoned mines (Humphrey and Kunz 1976). However, for many reasons, mines are becoming threatened refuges too. Since the 1980's, tremendous effort has been put into abandoned mine closure projects in several western states for public safety interests. However many have not been preceded by any biological surveys (Tuttle 1977; Navo 1993, 1994; Tuttle and Taylor 1994). In addition, many mines in the West are being reworked because new technology allows valuable minerals to be reclaimed from ore that was too poor to be mined economically in the past. Further, prices of some minerals (i.e. Uranium) has increased dramatically in recent years.

Weyandt et al. (2005) examined genetic diversity as measured by mtDNA and five microsatellites of the endangered *C. t. ingens*. The authors found that the observed genetic heterozygosity was lower than expected in this subspecies. However, this study was not able to compare this diversity to other *C. townsendii* populations, hence they were not able to determine if these estimates exemplified diversity within this species or were a reflection of a loss of diversity due to the disjunct and isolated distribution of this one subspecies (Weyandt et al. 2005). Our study sought to estimate genetic diversity and population demographic parameters of each of three sampled *C. townsendii* subspecies, *C. t. pallescens, C. t. townsendii*, and *C. t. virginianus*, from mtDNA and autosomal microsatellites and then to use a comparative approach to elucidate differences among the subspecies. We expected that when population demographics of the three subspecies were compared that the genetic consequences of genetic drift would be evident in the endangered *C. t. virginianus*. If genetic drift is driving diversity in *C. t. virginianus* populations, estimates of genetic diversity and effective population sizes should be significantly lower in these populations than in populations of the western *C. t. pallescens* and *C. t. townsendii*. Conversely, it may be the case that the entire species has similar genetic diversity estimates and effective population sizes. This case would suggest that either the isolated and disjunct populations of *C. t. virginianus* are not primarily influenced by genetic drift or the entire species is characterized by low genetic diversity.

Our study also sought to elucidate the degree of connectivity among remaining populations of the endangered *C. t. virginianus*. These populations are restricted and known from only five disjunct areas (Fig. 1): Pendleton, Grant, and Tucker counties, West Virginia (Bagley 1984) and Highland County, Virginia (bordering Pendleton County, West Virginia); Fayette County, West Virginia (this study); Tazewell County, Virginia (Bagley 1984); Lee County, Kentucky (Bagley 1984); and Avery County, North Carolina (Clark and Lee 1987). It is possible that because known colonies of *C. t. virginianus* (Bagley 1984) are in such disparate regions and these regions are outside the known dispersal distances of these bats (Humphrey and Kunz 1976), that these populations no longer maintain genetic connectivity. Such a scenario would mean that each regional population is an isolated entity and subject to genetic drift and inbreeding, which would seriously jeopardize the evolutionary potential of this unique lineage of *C. townsendii* (Piaggio and Perkins 2005).

Phylogenetic analyses of *C. townsendii* discovered that *C. t. pallescens* and *C. t. townsendii* meet in the Southern Rocky Mountains where they are sympatric in at least one area, Boulder County, Colorado (Piaggio and Perkins 2005). The largely discrete distributions of these subspecies with a small area of overlap fit distributions predicted by secondary contact (Marjoram and Donnelly 1994). Another goal of this study was to examine populations of both subspecies in this area of secondary contact in Colorado using a molecular approach to determine if there is currently gene flow between these two subspecies. A low degree of gene flow between subspecies is expected in areas of secondary contact (Smith et al. 1997). If gene flow occurs between these two subspecies as predicted, does it occur among all sampled populations or only a few? Also, will additional areas of sympatry be detected when more roosts in Colorado are sampled? This population level examination of populations of both subspecies in Colorado will also serve as an initial examination of genetic diversity, population differentiation, and population sizes of these presumed declining taxa and serve for comparison to *C. t. virginianus* sampled populations.

A final goal of this study was to test whether dispersal among populations of Townsend's big-eared bats is driven primarily through male dispersal. Male-biased dispersal is assumed to drive social structure within most mammals (Greenwood 1980; Dobson 1982). In such a system, females are philopatric and tend to be closely related within a population, while males disperse widely and act as vectors of gene flow. In this case, population structure estimated from maternally inherited genetic markers, such as mtDNA, will exhibit higher levels than estimates obtained from autosomal loci, which have a paternal contribution (Avise 1995). Bats are social animals (Bradbury 1977), and most studies examining dispersal using molecular data have demonstrated that they adhere to the mammalian model of male-biased dispersal (Wilmer et al. 1994; Petri et al. 1997; Burland et al. 1999; Petit and Mayer 1999; Wilmer et al. 1999; Kerth et al. 2000; McCracken and Wilkinson 2000; Petit and Mayer 2000; Petit et al. 2001). However, based on mark-recapture work, there are at least four exceptions to this dispersal model in bats. *Corynorhinus townsendii*

(Pearson 1952; Barbour and Davis 1969; Humphrey and Kunz 1976) of this study, and *C. rafinesquii* (Jones and Suttkus 1975; Menzel et al. 2001) in North America, *Plecotus auritus* in Europe (Entwistle et al. 2000), and *Miniopterus schreibersii natalensis* in South Africa (Miller-Butterworth et al. 2003), have all shown extreme philopatry of both sexes to winter and summer roosts, suggesting that neither males nor females disperse. Based on these studies, it is possible that *C. townsendii* does not exhibit male sex-biased dispersal. However Weyandt et al. (2005) found genetic evidence for male-biased dispersal in *C. t. ingens* based on significant differentiation of mitochondrial haplotypes among populations and a lack of differentiation of autosomal microsatellite loci. Therefore, we tested whether or not genetic evidence exists for male-biased dispersal in any of the three subspecies of *C. townsendii* examined in this study.

Methods

Sampling and DNA extraction

Tissue punches from individuals that were captured and released in Colorado from 2000–2005 were collected from 94 individuals (Table 1; Fig. 1). Further, 4 samples from *C. t. townsendii* from Wyoming were included because it appears the samples of *C. t. townsendii* within the range of *C. t. pallescens* have moved in either from western Colorado or from Wyoming (Piaggio and Perkins 2005). The 69 individuals sampled of the endangered subspecies, *C. t. virginianus*, were collected either as road kills, dead bats found in roosts over the past 15 years, or as tissue punches from wings of bats that were captured and released from 2000–2004 (Table 1, Fig. 1). Tissue punches are a 3mm tissue biopsy from the right wing of each animal (Wilmer and Barratt 1996), which were collected by biologists in the field and frozen or preserved in a 20% dimethyl sulfoxide, 0.25M EDTA, saturated with NaCl, pH 8.0 solution (Seutin et al. 1991). Genomic DNA from tissue was extracted from half of a wing punch or an equivalent sized piece of tissue from carcasses using a DNeasy Tissue Extraction Kit (Qiagen Inc., Valencia, CA) following the manufacturer's tissue extraction protocol.

DNA amplification, sequencing, and genotyping

The polymerase chain reaction (PCR) was used to produce amplified DNA fragments of the mtDNA control region and was carried out in a Mastercycler Thermalcycler (Eppendorf). Amplification and sequencing of the control region followed the procedures and protocols as detailed in Piaggio and Perkins (2005).

Individual bats were genotyped using six autosomal microsatellite loci: EF15B, EF20C, EF21, EF14 (Vonhof et al. 2002), NN8 (Petri et al. 1997), and PAUR 05 (Burland et al. 1998). We tested 15 microsatellite primer pairs developed from micro-chiropteran libraries that had shown some cross-species amplification in *C. townsendii* or a closely related species (Petri et al. 1997, Burland et al. 1998, Mayer et al. 2000, Storz 2000; Vonhof et al. 2002). Only six of these amplified reliably for the three *C. townsendii* subspecies. Products were amplified via PCR with one primer end-labeled with a TET, FAM, or HEX fluorescent label (Sigma-Genosys). Each microsatellite PCR was run in a standard 25 µl reaction, which contained optimized amounts of PCR water, 5X buffer C (Invitrogen), 2.5 µl of dNTP (10 mM, Invitrogen), 2.5 µl of each primer (1 pM/µl), *Taq* DNA polymerase (Promega), and 1 µl of genomic DNA. Amplification consisted of an initial denaturation at 94°C for 2 min followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 56°C (PAUR05 and EF15), 52°C (EF21), or 46°C (EF14, EF20C, and NN8) for 45 s, and extension at 72°C for 45 s with a final extension period of 7 min at 72°C. The software CONVERT (Glaubitz 2004) was used to translate genotyping data to formats used in downstream analyses.

Sequence analyses

The control region sequences were aligned first in Clustal X 1.81 (Thompson et al. 1997) and further aligned by eye using Sequencher (vers. 4.2.2 Genecodes Corporation). GenBank accession numbers of resulting sequences are recorded in Table 1.

Maximum parsimony (MP) and maximum likelihood (ML) phylogenetic analyses were carried out using PAUP* 4.0b (Swofford 2003). Because the western subspecies and the endangered *C. t. virginianus* are not sister taxa, samples of *C. t. pallescens* and *C. t. townsendii* from Colorado were analyzed together and *C. t. virginianus* samples were analyzed separately (Piaggio and Perkins 2005). Four samples of *Corynorhinus mexicanus* were used as an outgroup for both analyses because it is the closest sister taxon to *C. townsendii* (Hoofer and Van Den Bussche 2001; Piaggio and Perkins 2005). MP trees were produced using heuristic searches starting with an addition sequence of 100 replicates of random stepwise addition trees using unweighted parameters. Stability of nodes was determined through estimates of MP bootstrap support (Felsenstein 1985). The most appropriate model of evolution for the sequence dataset was determined by employing a hierarchical likelihood ratio test (LRT; Yang et al. 1994). When applicable, likelihood statistics were used to select one parsimony tree from the set of resulting most parsimonious trees, and this tree was then used as a starting tree to

Table 1 Samples sequenced and analyzed in this study with localities, donator/ownership, and GenBank accession numbers indicated

Taxon	Locality	Donor/owner	Pop	Acc No.
C. townsendii pallescens	Colorado, Boulder	Piaggio	A	AY713507; AY713530; AY713676–AY713677; AY713680–AY713681
	Colorado, Boulder	CDOW B/IMP	A	AY713743; AY713646–AY713650
	Colorado, Boulder	Lauren Golten	A	AY776016–AY776017
	Colorado, Teller	CDOW B/IMP	B	AY713744–AY713747
	Colorado, El Paso	CDOW B/IMP	B	AY713626–AY713627
	Colorado, Baca	CDOW B/IMP	C	AY713733–AY713734; AY713757–AY713766
	Colorado, Chaffee	Piaggio	D	AY776000–AY776005
	Colorado, Chaffee	CDOW B/IMP	D	AY776006
	Colorado, Fremont	CDOW B/IMP	E	AY776007–AY776015
	Colorado, Clear Creek	Piaggio		AY713732
	Colorado, Custer	CDOW B/IMP		AY713505
	Colorado, Larimer	CDOW B/IMP		EF636822
	Colorado, Mineral	CDOW B/IMP		AY713510; AY713683
C. townsendii townsendii	Colorado, Boulder	Piaggio	A	AY713506; AY713716
	Colorado, Boulder	CDOW B/IMP	A	AY776018
	Colorado, San Miguel	CDOW B/IMP	F	AY713527; AY713625 AY713702–AY713703; AY713644–AY713645; AY713678–AY713679; AY713866–AY713867
	Colorado, Montrose	CDOW B/IMP	G	AY713682; AY713736; AY713742; AY713748–AY713750; AY713862–

Table 1 continued

Taxon	Locality	Donor/owner	Pop	Acc No.
				AY713865; AY713868–AY713872
	Colorado, Garfield	Piaggio		AY713511
	Colorado, La Plata	CDOW B/IMP		AY713508–AY713509
	Colorado, Larimer	CDOW/BIMP		EF636823 EF636824 EF636825
	Colorado, Mesa	CDOW B/IMP		AY713512; AY713528–AY713529
	Colorado, Montrose	USGS Ernie Valdez EWV 1382		AY713526
	Colorado, Pitkin	Piaggio		EF636826 EF636827 EF636828
C. townsendii virginianus	West Virginia, Pendleton	WVDNR Craig Stihler	H	AY713533–AY713546; AY713548–AY713549; AY713551–AY713554
	West Virginia, Pendleton	WVDNR Craig Stihler	I	AY713550; AY713555; AY713735; AY713793
	West Virginia, Grant	WVDNR Craig Stihler	I	AY713547
	West Virginia, Fayette	WVDNR Craig Stihler	J	AY713737–AY713741
	Virginia, Tazewell	WVDNR Craig Stihler	K	AY713794–AY713816
	Kentucky, Lee	KDFWR Traci Wethington	L	AY713873–AY713875; AY713879; AY713883–AY713891
	Kentucky, Jackson	KDFWR Traci Wethington	L	AY713876
	Kentucky, Estill	KDFWR Traci Wethington	L	AY713880; AY713892
C. mexicanus	Durango, Mexico	CIIDIR CRD 3110 Celia López-González	–	AY713590

Table 1 continued

Taxon	Locality	Donor/owner	Pop	Acc No.
	Durango, Mexico	CIIDIR CRD 3125 Celia López-González	–	AY713591
	Durango, Mexico	CIIDIR CRD 3115 Celia López-González	–	AY713593
	Milpa Alta, Distrito Federal, Mexico	Rafael Avila-Flores	–	AY713785

Locality—state, county or state, country as applicable; Acc No.—GenBank Accession Number; Catalog numbers provided when possible. Donor/owner abbreviations are as follows: CDOW B/IMP—Colorado Division of Wildlife's Bats/Inactive Mines Project; CIIDIR—Colección Regional Durango (Vertebrados), CIIDIR Durango, Instituto Politécnico Nacional, México; KDFWR—Kentucky Department of Fish and Wildlife Resources; USGS—United States Geological Survey, Biological Resources Division; WVDNR—West Virginia Department of Natural Resources

generate a ML tree. This was achieved with the selected likelihood model and estimated parameters enforced.

Evaluation of populations

Trapping at individual roosts of *C. townsendii* in Colorado rarely resulted in more than one or two individuals being captured, however, there were some maternity roosts that provided seven to 12 individual captures. For subsequent analyses, two populations represent samples from single roosts (C and E). In other cases, populations are comprised of individuals from the same county or adjacent counties with capture localities located no more than 30 km apart (A, B, D, F, and G; Table 1, Fig. 1). This distance was applied because the longest distance migration between roosts documented in literature for these bats is 32.2 km (Pearson 1952). This is further supported by data from West Virginia where the greatest movement recorded between summer and winter roosts was 31.9 km (Stihler unpub. data). Some individuals that were included in the phylogenetic analyses were excluded from the population evaluations because they represented too few samples outside the 30 km radius limit of one of the populations mentioned above.

The third subspecies, *C. t. virginianus*, was sampled from four of the five geographic regions where roosts are clustered (H-L; Table 1, Fig. 1) excluding a North Carolina population. Some sampled roosts were lumped together and considered as one population (I, J, and L) because they were within a 30 km radius of one another. Other populations represent samples from a single roost (H and K).

Mitochondrial DNA population diversity analyses

Genetic diversity within populations of all three subspecies was described from mtDNA control region sequences as the number of individuals sequenced (N), number of unique haplotypes (H), haplotype diversity (h), nucleotide diversity (π) (Nei 1987), parsimony informative sites, and average pairwise differences between groups. A Mann-Whitney *U*-test (Sokal and Rohlf 1995) was used to assess statistical significance of differences in within-population genetic diversity measures, h and π, between the western subspecies and the endangered subspecies. Pairwise population structure, or differentiation, was estimated from mtDNA using F_{ST} (Weir and Cockerham 1984), and significance was determined by 5000 randomization tests. Populations were examined for an effect of isolation-by-distance (IBD) by testing the correlation between linearized F_{ST} values and straight-line pairwise geographic distances (Slatkin 1993, 1995). Distances were measured in kilometers from the center of one population (or roost) to another. Population-level analyses of mtDNA control region sequences were performed using Arlequin 2.0 (Schneider et al. 2000).

Autosomal microsatellite population diversity analyses

Microsatellite loci were tested for a significant departure from Hardy-Weinberg equilibrium (HWE) expressed as differences in expected heterozygosity (H_e) and observed heterozygosity (H_o) for each population at each locus using Arlequin 2.0 (Schneider et al. 2000). Bonferroni corrections were used to compute critical significance levels for multiple tests of these data (Rice 1989). Loci were also examined for evidence of null alleles using MICRO-CHECKER (Van Oosterhout et al. 2004). Null allele frequencies were calculated in MICRO-CHECKER with a 99% confidence interval (Brookfield 1996). Intracolonial genetic variability estimated from microsatellites is described as the mean number of alleles (A), allelic richness (a), and the number of private alleles (pa). Differences between the western subspecies and the endangered subspecies in average within-population diversity measures, A,

a, and H_e were tested for significance using Mann-Whitney *U*-tests (Sokal and Rohlf 1995). Pairwise comparisons of loci for linkage disequilibria in each population were carried out and inbreeding coefficients (F_{IS}) were calculated for each population. These analyses were either performed by hand or with FSTAT 2.9.3 (Goudet 2001).

Population structure was estimated by pairwise F_{ST} comparisons between populations from microsatellites (Weir and Cockerham 1984) and significance was ascertained by generating an expected distribution based on randomizations with Monte Carlo simulations in Arlequin 2.0 (Schneider et al. 2000). Bonferroni corrections were made to correct for multiple comparisons of these data (Rice 1989). An IBD test of microsatellite linearized population differentiation and geographic distance (Slatkin 1993) was employed in Arlequin 2.0.

To determine whether sex-biased dispersal occurs we followed the methods of Balloux et al. (1998) and Mossman and Waser (1999) where F_{ST} values estimated from mtDNA are compared to F_{ST} values estimated from autosomal microsatellite loci. Because mtDNA is inherited only from maternal lineages, it can be assumed that F_{ST} values estimated from this locus are due to the movement of females only. Conversely, microsatellites are biparentally inherited and if estimates of F_{ST} are different from the mtDNA estimates, differences are attributed to the movement of males.

Average *h* or gene diversity over all loci (Arlequin 2.0), was used to calculate effective population size (N_e) of each phylogroup, under the assumption of equilibrium using the equation, $N_e = h/4\ \mu(1–h)$ (Nei 1987). A mutation rate of 10^{-3} was assumed to be appropriate for these microsatellite loci (Weber and Wong 1993).

To test each population for evidence of a population bottleneck, the program BOTTLENECK (Cornuet and Luikart 1996) was used and an infinite alleles model was assumed with 9000 iterations. This program tests for signs of a recent reduction in N_e by detecting significant allelic modeshifts and heterozygosity alterations using a one-tailed Wilcoxon signed-rank test.

Results

Mitochondrial DNA variation and phylogenetic analyses

There were 56 unique control region haplotypes identified from the 94 individuals sequenced for the mtDNA control region from the two western subspecies sampled from Colorado, *C. t. pallescens* and *C. t. townsendii*. These sequences included populations A-G and 4 individuals from Wyoming of *C. t. townsendii*. Within the 1068 base-pair (bp) control region sequence fragment surveyed, 902 characters were constant, 42 variable characters were parsimony uninformative, and 124 variable characters were parsimony informative. Pairwise uncorrected genetic distances within both subspecies ranged from 0.00–0.02, and between the subspecies from 0.06–0.09. Unweighted maximum parsimony analyses (including the outgroup taxa and WY individuals) generated 3,464 equally most parsimonious trees with a length (L) of 442, a consistency index (CI) of 0.733, and a retention index (RI) of 0.963. The LRT demonstrated that the HKY85 plus the gamma shape parameter and invariable sites (HKY85 + I + G) model was a significantly better fit to the data than other models of evolution (Hasegawa et al. 1985). The parameters estimated for this model using the LRT include: *ti/tv* = 4.36; base composition A = 0.30, C = 0.28, G = 0.15, T = 0.26; gamma shape parameter $\alpha = 1.15$; invariable sites i = 0.55. The parsimony tree with the lowest log likelihood score (-ln *L* = 3768.123) was used to generate ML trees with parameters enforced.

One of the seven recovered ML trees is presented with MP bootstrap as support for major nodes (Fig. 2). Each of the two subspecies formed a well-supported, largely geographically distinct, monophyletic clade (Fig. 2); however, within each clade there was little resolution, which explained the differences in the seven recovered trees.

There were 69 individuals sequenced for the control region of the endangered subspecies, *C. t. virginianus* representing five populations (H-L). The *C. t. virginianus* sequences exhibited pairwise uncorrected distances ranging from 0.00–0.02 among populations, and provided 18 unique haplotypes. Maximum parsimony analyses produced four trees (L = 221; CI = 0.89; RI = 0.96) from 1065 bp of the control region, 877 of these characters were constant, 81 were variable but parsimony uninformative, and 107 sites were parsimony informative. A HKY85 + G model was selected under the LRT for the *C. t. virginianus* sequence data (*ti/tv* = 3.84; A = 0.31, C = 0.28, G = 0.15, T = 0.25; $\alpha = 0.27$). Under likelihood these parameters were enforced and likelihood trees were generated.

There were four ML trees generated (-ln *L* = 2517.510), the only differences were generated by different relationships among tip branches within one clade (K; Fig. 3). A ML tree is presented (Fig. 3) with MP bootstrap values on the nodes indicating support. Each of the four ML trees was identical to one of the four parsimony trees. There were four major clades inferred, all with little resolution within the clades. One of these clades was entirely unresolved and contains all but one individual of two populations (H and I) that were collected from the Ridge and Valley region of northeastern West Virginia. A sister relationship was inferred between the southern West Virginia (J) and northern Virginia populations (K), but this only has modest support. These two populations were then sister to a clade

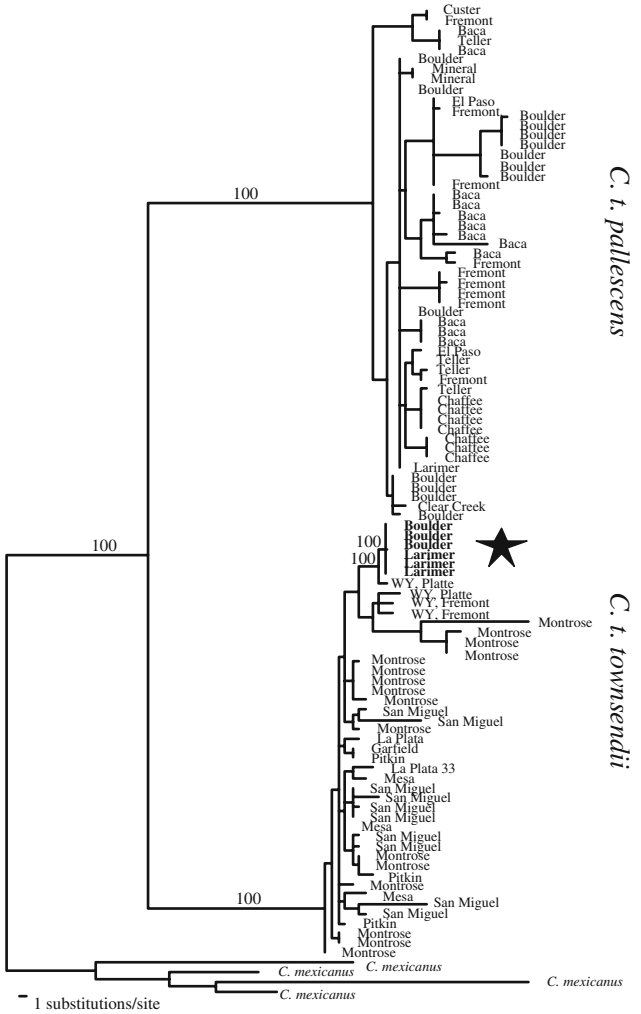

Fig. 2 Mitochondrial DNA ML phylogram of *C. t. pallescens* and *C. t. townsendii* from Colorado and *C. t. townsendii* from Wyoming. HKY + G + I model parameters are enforced. Clades corresponding to subspecies are noted. Support for nodes are shown as MP bootstrap. Individuals are labeled with the name of the county where they were collected. The individuals from Boulder County and Larimer County, Colorado, which were collected within the range of *C. t. pallescens*, that have *C. t. townsendii* haplotypes, are identified in bold and marked with a star

that contains all of the Kentucky samples (L) and one sample from the Ridge and Valley population (H) of northeastern West Virginia (Fig. 3).

Sequence diversity among populations

Sequence diversity of all three subspecies is shown per population in Table 2. Both haplotype diversity and nucleotide diversity were significantly lower (Mann-Whitney *U*-test $P < 0.05$) in the endangered *C. t. virginianus* than in the two western subspecies, *C. t. pallescens* and *C. t. townsendii*. Overall, the range of pairwise differences was lower within *C. t. virginianus* than within *C. t. pallescens* or *C. t. townsendii* (Table 3).

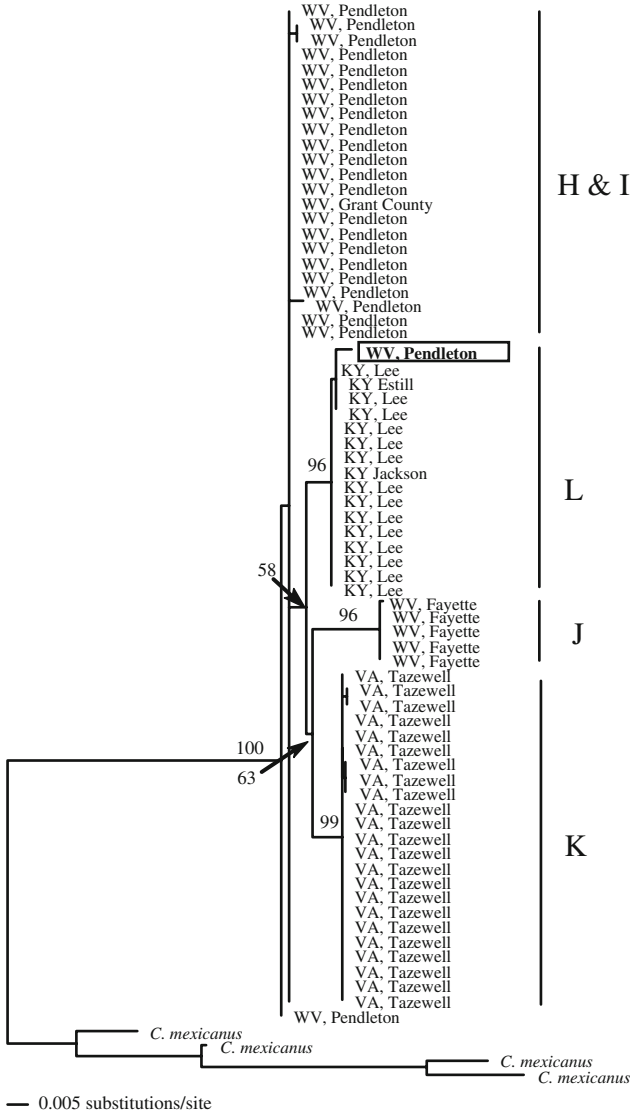

Fig. 3 Mitochondrial DNA ML phylogram of *C. t. virginianus* samples. HKY + G model parameters are enforced. Support for nodes are shown as MP bootstrap. Clades corresponding to populations are labeled (H–L), and individuals are labeled by state and county of capture. The individual from population H collected in Pendleton County, West Virginia with a haplotype of population L, Kentucky, is shown in bold

The level of mtDNA population differentiation between populations within all three subspecies was measured by pairwise estimates of F_{ST} (Table 3). All *C. t. pallescens* populations were significantly differentiated from populations from both *C. t. townsendii* populations. Neither of the *C. t. townsendii* populations were significantly differentiated from one another. Only two pairwise comparisons out of 10 were significant among *C. t. pallescens* populations. All populations of *C. t. virginianus* were significantly differentiated from one another except two geographically proximate populations collected from the Ridge and Valley region of West Virginia (H and I).

Table 2 Sequence diversity measures among populations of three *C. townsendii* subspecies, *C. t. pallescens* (A–D), *C. t. townsendii* (F, G), and *C. t. virginianus* (H–L) estimated from mtDNA

Population	N	H	h	SE	π	SE	PI
A	17	13	0.963	0.033	0.026	0.013	77
B	6	6	1.000	0.096	0.008	0.005	3
C	12	10	0.970	0.044	0.007	0.004	17
D	7	2	0.571	0.120	0.003	0.002	5
E	9	6	0.833	0.127	0.008	0.005	11
F	10	9	0.978	0.054	0.006	0.004	5
G	15	10	0.943	0.040	0.005	0.003	15
H	14	4	0.396	0.159	0.002	0.001	1
I	11	4	0.491	0.176	0.001	0.001	0
J	5	3	0.800	0.164	0.009	0.001	0
K	23	3	0.170	0.103	0.001	0.001	0
L	16	4	0.517	0.132	0.001	0.001	1

Diversity statistics are as follows: N, number of individuals sequenced; H, number of unique haploytpes; h, haplotype diversity; π, nucleotide diversity; SE, standard error; PI, parsimony informative sites

Table 3 Summary of mitochondrial DNA sequences pairwise differences between populations, within a population, and pairwise F_{ST} estimates between populations

Pop	A	B	C	D	E	F	G
A	26.90	21.61	21.28	20.12	21.25	55.58	55.43
B	0.10	9.00	10.36	7.67	10.70	65.78	65.72
C	0.16	0.20	7.82	8.25	9.94	65.33	65.37
D	0.16	0.20	0.28*	3.42	8.52	60.71	60.93
E	0.11	0.15	0.15	0.24*	9.19	64.80	64.87
F	0.66*	0.88*	0.89*	0.91*	0.88*	6.80	6.59
G	0.70*	0.91*	0.91*	0.93*	0.90*	0.13	4.80

Pop	H	I	J	K	L
H	2.31	1.61	11.77	14.01	10.05
I	−0.02	0.98	14.06	14.16	10.50
J	0.83*	0.92*	1.00	12.29	12.98
K	0.93*	0.97*	0.98*	0.13	15.63
L	0.86*	0.92*	0.94*	0.98*	0.65

Above the diagonal are mtDNA sequences pairwise differences between populations, the diagonal are pairwise differences within a population, and below the diagonal are mtDNA pairwise F_{ST} estimates between populations. Populations of *C. t. pallescens* (A–E), *C. t. townsendii* (F–G), and *C. t. virginianus* (H–L) are shown

* Significant pairwise F_{ST} comparisons at the $P < 0.05$ level after sequential Bonferroni corrections (Rice 1989)

A test of the effect of IBD was performed on *C. t. pallescens* and *C. t. virginianus* populations, but not on *C. t. townsendii* because we have data from only two populations. There was no correlation of mtDNA pairwise population differentiation and pairwise geographic distances among populations of *C. t. pallescens* ($P = 0.79$) or *C. t. virginianus* ($P = 0.62$).

Genotypic variability

Of the six microsatellite loci used to genotype *C. townsendii* samples, one (NN8) was monomorphic in this species, so only five were used in the subsequent analyses. Tests for HWE across all microsatellite loci in all populations of all three subspecies indicated several significant departures from HWE after sequential Bonferroni corrections. Departures were due to four significant deficiencies and a single significant excess (Table 4) all found in the western subspecies. In spite of this, no locus showed significant deviations from HWE in more than one population per subspecies so all loci were retained. Deficiencies of heterozygotes may be produced by the presence of null alleles. In fact, evidence of null alleles detected by MICRO-CHECKER explained two of the five violations of HWE (Table 4), both in population G of *C. t. townsendii* in loci PAUR05 (Brookfield 1 frequency = 0.15) and EF21 (Brookfield 1 frequency = 0.18). Null alleles were also detected at locus PAUR05 (Brookfield 1 frequency = 0.11) in population A (Table 4). We did not drop these loci because null alleles were only detected in two of twelve comparisons at PAUR05 and in one population at EF21. Further, in the current study, there was an absence of non-amplifying individuals across all loci, presence is indicative of a null allele problem. Additionally, Weyandt et al. (2005) also used PAUR05 and EF21 to evaluate populations of the closely-related, federally endangered *Corynorhinus townsendii ingens* and did not find violations of HWE or evidence of null alleles. Finally, the populations where null alleles and/or HWE violations were identified included multiple individuals from maternity roosts, therefore having closely related individuals in these samples could have contributed to the apparent homozygosity excess (Bourgain et al. 2004).

In pairwise tests of linkage equilibrium, there was no evidence of loci being linked. Summary statistics for microsatellite genetic variation are in Table 5. There was a significantly lower level of diversity found in *C. t. virginianus* than in *C. t. townsendii* and *C. t. pallescens* as measured by H_e, average number of alleles per locus (A), and average allelic richness per population (a; Mann-Whitney *U*-test, $P < 0.05$). The estimates of F_{IS} (Table 6) were significantly different from zero in only two populations; one population of *C. t. townsendii* (G) and one of the *C. t. virginianus* populations from the Ridge and Valley region of West Virginia (I).

Microsatellite loci revealed significant but low F_{ST} estimates in two of 21 pairwise population comparisons within and between the two western subspecies (Table 6). Among populations of the subspecies *C. t. pallescens* there was a single F_{ST} comparison that showed significant

Table 4 Results from HWE

Pop	EF15		PAUR05		EF21		EF20		EF14	
	H_o	H_e	H_o	H_e	H_o	H_e	H_o	H_e	H_o	H_e
A	0.75	0.92	0.65°	0.92	0.87	0.86	0.13	0.18	0.93	0.83
B	0.80	0.89	1.00	0.97	0.83	0.92	0.17	0.32	0.67	0.82
C	0.67*	0.87	0.92*	0.76	0.58*	0.87	0.58	0.43	1.00	0.84
D	1.00	0.98	0.43	0.89	0.71	0.80	0.29	0.38	0.86	0.81
E	1.00	0.93	1.00	0.94	1.00	0.90	0.22	0.31	0.89	0.88
F	1.00	0.96	1.00	0.95	0.44	0.88	0.10	0.10	0.70	0.68
G	0.67	0.86	0.60*°	0.924	0.40*°	0.78	–	–	0.67	0.75
H	0.54	0.73	0.67	0.63	0.69	0.82	0.17	0.36	0.33	0.52
I	0.70	0.79	0.56	0.80	0.50	0.86	0.57	0.58	0.40	0.73
J	0.40	0.87	0.60	0.78	1.00	0.71	–	–	0.60	0.78
K	0.74	0.73	0.78	0.82	0.68	0.77	0.17	0.16	0.43	0.45
L	0.69	0.76	0.69	0.69	1.00	0.78	0.13	0.18	0.50	0.49

Expected heterozygosity (He) and observed heterozygosity (Ho) for each locus across each population. Populations of *C. t. pallescens* (A–E), *C. t. townsendii* (F, G), and *C. t. virginianus* (H–L) are shown

* Significant departures from HWE ($P < 0.05$) after sequential Bonferroni corrections (Rice 1989), ° null alleles detected with 99% confidence interval as described (Brookfield 1996), and – indicates monomorphic locus

Table 5 Summary statistics of genetic diversity generated from microsatellite DNA for populations of *C. t. pallescens* (A–E), *C. t. townsendii* (F, G), and *C. t. virginianus* (H–L)

Pop	EF15			PAUR05			EF21			EF20			EF14		
	A	a	pa	A	a	pa	A	a	pa	A	a	pa	A	a	pa
A	12	6.91	1	12	6.91	0	12	6.04	1	3	1.63	0	8	5.23	0
B	6	6.00	0	10	8.64	0	8	7.14	0	2	1.83	0	5	4.79	0
C	8	5.61	0	6	4.42	0	6	5.10	0	2	1.99	0	7	5.31	0
D	11	9.32	0	6	5.34	0	7	5.56	0	2	1.93	0	5	4.63	0
E	10	7.21	0	10	7.35	1	9	6.75	0	2	1.82	0	7	5.61	0
F	15	8.48	3	9	7.96	1	6	5.21	0	2	1.50	0	6	4.29	0
G	10	6.00	0	12	7.16	0	7	4.36	0	1	1.00	0	7	4.37	1
All	23	7.56	4	18	7.21	2	13	5.81	1	3	1.77	0	11	5.14	1
H	5	4.01	0	4	3.09	0	6	4.81	2	2	1.91	0	2	2.00	0
I	6	4.52	0	5	4.34	0	6	4.73	1	3	2.87	1	5	3.98	2
J	5	5.00	1	3	3.00	1	3	3.00	0	1	1.00	0	3	3.00	0
K	5	3.86	0	7	4.85	1	6	4.31	0	2	1.64	0	3	2.61	0
L	8	4.57	1	3	2.98	0	6	4.41	1	2	1.53	0	2	2.00	0
All	10	5.29	2	10	5.61	2	9	4.61	2	3	1.80	1	5	2.89	2

Diversity is measured as: A, number of alleles; a, allelic richness; pa, private alleles

differentiation between populations. Both populations of *C. t. townsendii* lacked differentiation from one another as estimated from microsatellite data. Further, some pairwise comparisons lacked differentiation between populations of *C. t. pallescens* and *C. t. townsendii*.

Only three of 10 pairwise F_{ST} comparisons were not significant among populations of *C. t. virginianus* (Table 6). These two populations were the same populations that were not differentiated according to mtDNA F_{ST} comparisons (H and I). Further populations H and I were not significantly differentiated from population J.

Population differentiation (F_{ST}) estimated from microsatellite DNA was tested for correlation to geographic distances between populations. A low but significant correlation exists among populations of *C. t. pallescens* ($P = 0.05$) and a significant correlation exists among *C. t. virginianus* populations ($P = 0.03$). Therefore, a model of IBD may influence the genetic structure of *C. t. pallescens*

Table 6 Inbreeding coefficient (F_{IS}) estimated and estimates of pairwise population structure (F_{ST}) from microsatellite DNA for each population of *C. t. pallescens*, *C. t. townsendii*, and *C. t. virginianus*

Pop	A	B	C	D	E	F	G
A	0.11	–	–	–	–	–	–
B	0.003	0.04	–	–	–	–	–
C	0.06*	0.06	−0.01	–	–	–	–
D	0.02	0.01	0.08	0.13	–	–	–
E	0.02	0.01	0.03	0.02	−0.08	–	–
F	0.000	0.01	0.04	0.04	0.02	0.09	–
G	0.02	0.04	0.09*	0.04	0.06	0.000	0.29*

Pop	H	I	J	K	L
H	0.18	–	–	–	
I	0.00	0.23*	–	–	
J	0.10	0.07	0.13	–	
K	0.15*	0.16*	0.15*	0.03	
L	0.14*	0.12*	0.11*	0.10*	−0.06

F_{IS} is shown on the diagonal and F_{ST} comparisons are below the diagonal. Populations are of *C. t. pallescens* (A–E), *C. t. townsendii* (F, G), and *C. t. virginianus* (H–L)

* Significant deviations from random ($P < 0.05$) after sequential Bonferroni corrections (Rice 1989)

and *C. t. virginianus* populations. However, for *C. t. pallescens* when the most geographically distant and disjunct population (Fig. 1), Population C, was removed from the IBD test the results were no longer significant ($P = 0.24$). Population C was the only population of *C. t. pallescens* that is significantly differentiated from all other populations.

The range of N_e for each subspecies was as follows: *C. t. pallescens*, 420–813; *C. t. townsendii*, 382–460; and *C. t. virginianus*, 293–642 (Table 7). A population bottleneck was indicated in two of the six populations of *C. t. pallescens* (C and E) and in four of the *C. t. virginianus* populations (H, I, J, and K). Population bottlenecks in some populations (E and J) were interpreted in these populations from significant allelic modeshifts and significant heterozygosity alterations based on a one-tailed Wilcoxon signed-rank test. In the other populations (C, H, I, and K) one of the two tests was significant. It is notable that any populations showed evidence of a bottleneck, because the tests in BOTTLENECK require at least four variable loci with fewer than 20 loci being considered too small to provide power to discriminate patterns of bottleneck in populations.

Discussion

Genetic diversity

A significantly lower degree of genetic diversity has been identified in populations of the endangered subspecies, *C. t. virginianus* as inferred from both mtDNA and microsatellite DNA in this study. This is not surprising considering the small population sizes and reduced range of *C. t. virginianus* (USFWS 1979). This reduced genetic diversity means that genetic drift may be driving diversity within these populations and the biodiversity and evolutionary potential of *C. t. virginianus* has been diminished.

Genetic diversity within *C. t. townsendii* and within *C. t. pallescens* was not significantly different from each other. Although there have been concerns over the decline of populations of these western subspecies, their genetic diversity is not low. In *C. t. townsendii* population G there was a significantly high level of inbreeding and two loci (PAUR05 and EF21) had significant deviations from HWE with evidence of null alleles. This level of inbreeding and loss of heterozygosity is intriguing, especially because this is the most widespread subspecies. When this population is examined more closely, more than half (8 of 15) of the individuals in population G are from a single roost, which when analyzed alone have significantly high levels of inbreeding and consistently lower levels of genetic diversity than other populations. Therefore, the inbreeding found in this roost may account for the significant level of inbreeding and homozygosity found in population G. Higher sample sizes per roost, comparison of individual roosts on a microgeographic scale, and analyses with a greater number of microsatellites are required to unravel the reasons for this level of inbreeding.

Population structuring and connectivity

Among *C. t. virginianus* populations the lack of significant differentiation between populations H and I was not surprising because they occupy the same geographical region. Further, banding data collected by the West Virginia

Table 7 Effective population size (N_e) estimated from microsatellite DNA and expected heterozygosity across loci (*h*) for populations of *C. t. pallescens* (A–E), *C. t. townsendii* (F, G), and *C. t. virginianus* (H–L)

	A	B	C	D	E	F	G	H	I	J	K	L
h	0.627 ± 0.408	0.724 ± 0.444	0.714 ± 0.419	0.714 ± 0.433	0.765 ± 0.451	0.605 ± 0.383	0.648 ± 0.382	0.540 ± 0.330	0.720 ± 0.441	0.591 ± 0.382	0.566 ± 0.337	0.564 ± 0.354
N_e	420.24	655.80	626.32	626.32	813.83	382.91	460.23	293.48	642.86	361.25	326.04	323.39

Division of Natural Resources over the past 20 years demonstrates that some *C. t. virginianus* bats found roosting in four separate summer roosts are observed to hibernate primarily in a single roost in Pendleton County, West Virginia. Some individuals from two of the four summer roosts were found in hibernaculum 2 km away. Clearly, multiple summer roosts congregate within hibernacula in this area. Further, these banding data were the first piece of evidence that *C. t. virginianus* cross the continental divide to hibernate because some of the summer roosts are on the west side of the Allegheny Front and the hibernacula are on the east side of the Allegheny Front. Population structuring (F_{ST}) estimated from mtDNA (Table 3) showed significant levels of differentiation among populations of *C. t. virginianus* located in different geographical regions (H/I, J, K, and L; Fig. 1). Population structuring estimated from microsatellites (Table 6) showed this same differentiation among the regional populations except H/I to J. These levels of regional differentiation suggest a complete loss of connectivity among regional populations of *C. t. virginianus* among females and among males except between the northeastern and central West Virginia regions. The lack of significant effects of IBD estimated from mtDNA may confirm this loss because it suggests another cause of differentiation besides geographical distance (i.e. population isolation). However, evidence of significant effects of IBD was detected from microsatellite data, but this could be due to differences in inheritance modes. Further, microsatellite data support loss of connectivity of these regional populations through evidence of population bottlenecks and inbreeding in some populations of *C. t. virginianus*.

The mtDNA phylogeny inferred from *C. t. virginianus* has four clades, which are principally made up of members from each of the four geographically isolated regions, with one exception. There is an individual from the Ridge and Valley region of northeastern West Virginia that groups within the Kentucky clade. This can be explained by contamination, introgression, or shared ancestral haplotypes. In this case, contamination is unlikely because the West Virginia samples were processed in the lab before the Kentucky samples were received. This leaves introgression and shared ancestral polymorphism to explain this anomaly. Due to large geographical distances between these regions and the high degree of substructuring estimated from mtDNA, it is more parsimonious to conclude that the West Virginia sample that is well supported within the Kentucky clade represents an ancestral haplotype shared between these two populations (Fig. 2). The high statistical support of the regional populations as clades in the mtDNA phylogeny suggest that isolation of these regions was not a recent event.

One approach to aid conservation and management through genetic data is to identify Evolutionary Significant Units (ESUs) as conceived by Ryder (1986) and Moritz (1994). These ESUs are defined as phylogeographic subdivisions that have a recent common history, are genetically cohesive, and are isolated, lacking gene flow with other populations. The loss of genetic diversity within *C. t. virginianus*, the degree of separation and significant population differentiation among regional populations, and low effective population sizes, leads us to conclude that each region investigated in this study (Lee, Estill, and Jackson counties, Kentucky; Tazewell County, Virginia; Fayette County, West Virginia; and Pendleton and Grant counties, West Virginia) should be considered as separate ESUs and managed as such. Further, the remaining North Carolina population should be sampled and the genetic diversity of that population and its connectivity to the populations in this study should be assessed to determine if it should also be considered as a separate ESU. Although populations of *C. t. virginianus* have shown increases in roost membership (Bagley 1984) and a new population was identified recently (Fayette County, West Virginia), estimates of effective population sizes range from only 323–936 ($N_e = 936$ is a combination of N_e estimates for H and I, which based on their lack of differentiation and close geographic proximity, should be considered a single ESU; Table 7) in each ESU. If each region is considered as a separate ESU, then Tazewell County, Virginia has the lowest overall genetic diversity with mtDNA haplotypes approaching fixation, whereas the Ridge and Valley region of West Virginia has the highest overall genetic diversity. Therefore, these data can be used directly to prioritize conservation of these four ESUs. Nevertheless, each of these ESUs requires protection because they represent the remaining evolutionary potential of these bats.

Population structure estimated from mtDNA pairwise F_{ST} comparisons of populations of *C. t. townsendii* and *C. t. pallescens* showed significant population structuring between the subspecies. In contrast, nine of ten pairwise F_{ST} comparisons between a *C. t. pallescens* population and a *C. t. townsendii* population estimated from microsatellite DNA were not significant. Therefore, the hypothesis that *C. t. townsendii* and *C. t. pallescens* experience low levels of genetic exchange among a few populations in areas of secondary contact in Colorado is supported by microsatellite data. Further, this suggests that males may be responsible for dispersal in this system.

Gene flow between the two western subspecies is not supported by mtDNA in pairwise F_{ST} comparisons. However, evidence from mtDNA of gene flow between the two subspecies inferred from the phylogeny exists. Three males collected well within the range of *C. t. pallescens*, in Boulder, Colorado and one male and two females caught in Larimer County, Colorado are shown as *C. t. townsendii* haplotypes (Fig. 2). The current study identified an additional area of sympatry (Larimer County, Colorado) not

detected in a previous study of mtDNA from samples of *C. t. townsendii* and *C. t. pallescens* (Piaggio and Perkins 2005). These individuals were caught in four different years at six different mines, and all had identical control region sequence haplotypes. These samples grouped with a 100 bootstrap support with a *C. t. townsendii* individual from WY and grouped to a larger clade with WY individuals and individuals from western Colorado, Montrose County. In fact the alliance of these samples to WY suggests the direction from which these samples might have arrived. The introgression of *C. t. townsendii* males could suggest that they move into the range of *C. t. pallescens* in the fall of each year for breeding purposes. This is supported by the lack of microsatellite DNA population structure between these subspecies because these markers are bi-parentally inherited and by the fact that each of the four of the aberrant samples were males that were caught in the fall of four different years. However, in this scenario it is difficult to explain how each of these bats would have identical haplotypes and that two females are included in these samples. It cannot be that the same male was captured each year, because each sample has a different microsatellite genotype. It is possible that these are all offspring of one *C. t. townsendii* mother, which somehow came to reside in a *C. t. pallescens* maternity roost. It may also be possible that the signal seen in our data may represent both current gene flow among populations of *C. t. pallescens* and *C. t. townsendii* as evidenced by microsatellite data and past secondary contact as evidenced by the single mtDNA haplotype of *C. t. townsendii* found in the range of *C. t. pallescens*. It is difficult to conclusively select one of these explanations, but it is clear that there is or has been movement of a *C. t. townsendii* haplotype(s) from western Colorado into Wyoming and then into the northern Front Range of Colorado, which is the range of *C. t. pallescens* haplogroups.

It is not unprecedented for bats from two different phylogeographical maternal lineages to be found together in roosts along the northern Front Range of Colorado. In a study of big brown bats, *Eptesicus fuscus*, Turmelle (in prep.) and Neubaum et al. 2007 two maternal lineages were identified within Colorado: one found primarily in western North America and the other in the East. These haplogroups show 8.9% sequence divergence from each other and yet both forms were found in roosts in Larimer County, Colorado, which is adjacent to Boulder County to the north. In conclusion, it is clear that some process is allowing divergent maternal lineages of bats to have secondary and/or possibly continuing contact in this region of Colorado, but further study is required to elucidate the process.

Although neither western subspecies *C. t. pallescens* or *C. t. townsendii* shows signs of a reduction in population genetic diversity there still should be continued monitoring of population trends of these bats. In particular, *C. t. pallescens*, which occupies an area where extensive human population growth is occurring and where two of the populations showed evidence of a population bottleneck, should be monitored. Neither of these subspecies is abundant in Colorado as evidenced by low effective population sizes and by census data from the Colorado Division of Wildlife's Bats/Inactive Mines Project. In approximately 4,000 mines surveyed over the last 16 years of the project's tenure, only 10 maternity roosts (five of *C. t. pallescens* and five of *C. t. townsendii*) have been identified. Also only two hibernacula with more than 25 individuals and only one with more than 100 individuals have been found. Small population sizes suggest that western *C. townsendii* could, in the near future, exhibit the same signs of loss of genetic diversity as *C. t. virginianus*. Further, there may be reason for concern with directional introgression of the more widely distributed *C. t. townsendii* into the restricted *C. t. pallescens*.

Sex-biased dispersal

Pairwise estimates of F_{ST} from microsatellite DNA were much lower and fewer comparisons (2/21) that demonstrated significant differentiation between populations than estimates from mtDNA (12/21) in all the western subspecies (Table 3, Table 6). This difference is may be explained by the four-fold lower effective population size of mtDNA compared to autosomal DNA, or may be due to male biased dispersal in the western subspecies. In *C. t. virginianus* both classes of markers indicated almost complete population differentiation among geographic regions of roosts. Microsatellite population differentiation estimates differed from mtDNA estimates only by showing a lack of differentiation between the regional populations H/I and J. This suggests that either neither sex of *C. t. virginianus* disperses across regions or that males may disperse in some cases where females do not. One possible process that may explain the low signal of sex-biased dispersal in *C. townsendii* is that males and females are indeed philopatric to summer and winter roosts and males mediate gene flow by intermixing with other populations in transient roosts in between leaving summer roosts and moving to hibernacula, as has been found in two other bat species, *Plecotus auritus* (Burland et al. 1999) and *Miniopterus screibersii natalensis* (Miller-Butterworth et al. 2003). Another possibility is that, in reality, these bats practice a more complicated breeding scenario than suggested our test of sex-biased dispersal and the sampling approach used in this or other studies. For example, Stihler et al. (1997) documented a large increase in numbers of bats in late summer/early fall at a bachelor colony of *C. t. virginianus*. When a capture survey was conducted the population increase could be attributed to a sharp increase in numbers of females. Therefore, the breeding behavior of

this species is probably more complicated then we can infer from our data and requires further detailed study.

Conservation implications

Corynorhinus townsendii townsendii has been identified through mark-recapture studies as a relatively sedentary species across its range (Humphrey and Kunz 1976; Kunz and Martin 1982; Pierson et al. 1999). Further, radio-tracking from the ground in the western U.S. indicates that neither males nor females disperse farther than 30 km from a roost to a foraging area (Fellers and Pierson 2002) and the longest distance recorded for migration between seasonal roosts was 32.2 km (Pearson et al. 1952). However, our data suggest gene flow between *C. t. pallescens* and *C. t. townsendii* roosts that are at least 310 km apart, which may indicate longer distance movements than previously identified. Further, recent studies of maternity colonies have shown that they may occupy multiple roosts in an area where more than one underground feature is available (Sherwin et al. 2000a; Sherwin et al. 2000b; Sherwin et al. 2003) and recent data collected from radio-tracking from planes shows that a pregnant *C. townsendii* can travel over 150 km in a night of foraging (R. Sherwin, pers. comm.). Together, these data suggest that *C. townsendii* (in the west) can and do move longer distances than initially thought. Thus, we argue that conservation efforts should not assume that maternity colonies or hibernacula utilize a single roost for the season, or that roosts will be found only in tightly clustered geographical areas. Further, if habitat corridors are being planned for these bats, they may need to include larger areas than once thought. Finally, we recommend further research, maintenance of conservation efforts, and population monitoring to protect the remaining genetic diversity and evolutionary potential of *C. townsendii* populations.

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References

Avise JC (1995) Mitochondrial DNA polymorphism and a connection between genetics and demography of relevance to conservation. Conserv Biol 9:686–690

Bagley FM (1984) A recovery plan for the Ozark big-eared bat and the Virginia big-eared bat. US Fish and Wildlife Service, Twin Cities, Minnesota

Balloux F, Goudet J, Perrin N (1998) Breeding system and genetic variance in the monogamous, semi-social shrew, *Crocidura russula*. Evolution 52:1230–1235

Barbour RW, Davis WH (1969) Bats of America. University of Kentucky Press, Lexington, Kentucky

Bourgain C, Abney M, Schneider D, Ober C, McPeek MS (2004) Testing for Hardy-Weinberg Equilibrium in samples with related individuals. Genetics 168:2349–2361

Bradbury JW (1977) Social organization and communication. In: Wimsatt WA (eds) Biology of Bats, vol 3. Academic Press, New York. pp 1–72

Brookfield JFY (1996) A simple new method for estimating null allele frequency from heterozygote deficiency. Mol Ecol 5:453–455

Burland TM, Barrrat EM, Racey PA (1998) Isolation and characterization of microsatellite loci in the brown long-eared bat, *Plecotus auritus*, and cross-species amplification within the family Vespertilionidae. Mol Ecol 7:133–140

Burland TM, Barratt EM, Beaumont MA, Racey PA (1999) Population genetic structure and gene flow in the gleaning bat, *Plecotus auritus*. Proc R Soc Lond B Biol Sci 266:975–980

Burland TM, Wilmer JW (2001) Seeing in the dark: molecular approaches to the study of bat populations. Biol Rev 76:389–409

Clark MK, Lee DS (1987) Big-eared bat, *Plecotus townsendii*, in western North Carolina. Brimleyana 13:137–140

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001–2014

Dobson FS (1982) Competition for mates and predominant juvenile male dispersal in mammals. Anim Behav 3:1183–1192

Entwistle AC, Racey PA, Speakman JR (2000) Social and population structure of a gleaning bat, *Plecotus auritus*. J Zool (Lond) 252:11–17

Fellers GM, Pierson ED (2002) Habitat use and foraging behavior of Townsend's big-eared bat (*Corynorhinus townsendii*) in coastal California. J Mammal 83:167–177

Felsenstein J (1985) Confidence limits on phylogenies: an approach using bootstrap. Evolution 39:783–791

Glaubitz JC (2004) CONVERT: a user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. Mol Ecol Notes 4:309–310

Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Updated from Goudet (1995). Available from http://www.unil.ch/izea/softwares/fstat.html

Greenwood PJ (1980) Mating systems and dispersal in birds and mammals. Anim Behav 28:1140–1162

Handley CO (1959) A revision of the American bats of the genera *Euderma* and *Plecotus*. Proc U S Natl Mus 110:95–246

Hasegawa M, Kishino H, Yano T (1985). Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 22:160–174

Hoofer SR, van den Bussche RA (2001) Phylogenetic relationships of plecotine bats and allies based on mitochondrial ribosomal sequences. J Mammal 82:131–137

Humphrey SR, Kunz TH (1976) Ecology of a Pleistocene relict, the western big-eared bat (*Plecotus townsendii*) in the southern Great Plains. J Mammal 57:470–494

Jones C, Suttkus RD (1975) Notes on the natural history of *Plecotus rafinesquii*. Occas Pap Mus Zool LA State Univ 47:1–14

Kerth G, Mayer F, Konig B (2000) Mitochondrial DNA (mtDNA) reveals that female Bechstein's bats live in closed societies. Mol Ecol 9:793–800

Kunz TH, Martin RA (1982) Plecotus townsendii. Mamm Species 175:1–6

Marjoram P, Donnelly P (1994) Pairwise comparisons of mitochondrial DNA sequences in subdivided populations and implications for early human evolution. Genetics 136:673–683

Mayer F, Schlotterer C, Tautz D (2000) Polymorphic microsatellite loci in vespertilionid bats isolated from the noctule bat *Nyctalus noctula*. Mol Ecol 9:2208–2212

McCracken GF, Wilkinson GS (2000) Bat mating systems. In: Crichton EG, Krutzsch PH (eds) Reproductive biology of bats. Academic Press, San Diego, California, pp 321–362

Menzel MA, Menzel JM, Ford WM, Edwards JW, Carter TC, Churchill JB, Kilgo JC (2001) Home range and habitat use of male Rafinesque's big-eared bats (*Corynorhinus rafinesquii*). Am Midl Nat 145:402–408

Miller-Butterworth CM, Jacobs DS, Harley EH (2003) Strong population substructure is correlated with morphology and ecology in a migratory bat. Nature 424:187–191

Moritz C (1994) Defining 'evolutionary significant units' for conservation. Trends Ecol Evol 9:373–375

Mossman CA, Waser PM (1999) Genetic detection of sex-biased dispersal. Mol Ecol 8:1063–1067

Navo KW (1993) Update on Colorado's bats/inactive mines project. Bat Res News 34:69

Navo KW (1994) Guidelines for the survey of caves and abandoned mines for bats in Colorado. Colorado Division of Wildlife, Monte Vista, CO

Nei M (1987). Molecular evolutionary genetics. Columbia University Press, New York

Neubaum MA, Douglas MR, Douglas ME, O'Shea TJ (2007) Molecular ecology of the big brown bat (*Eptesicus fuscus*): genetic and natural history variation in a hybrid zone. J Mammal 88:1230–1238

Pearson OP, Koford MR, Pearson AK (1952) Reproduction of the lump-nosed bat (*Corynorhinus rafinesquei*) in California. J Mammal 33:273–320

Petit E, Mayer F (1999) Male dispersal in the noctule bat (*Nyctalus noctula*): where are the limits? Proc R Soc Lond B Biol Sci 266:1717–1722

Petit E, Mayer F (2000) A population genetic analysis of migration: the case of the noctule bat (*Nyctalus noctula*). Mol Ecol 9:683–690

Petit E, Balloux F, Goudet J (2001) Sex-biased dispersal in a migratory bat: a characterization using sex-specific demographic parameters. Evolution 55:635–640

Petri B, Pääbo S, Von Haeseler A, Tautz D (1997) Paternity assessment and population subdivision in a natural population of the larger mouse-eared bat *Myotis myotis*. Mol Ecol 6:235–242

Piaggio AJ, Perkins SL (2005) Molecular phylogeny of North American long-eared bats (Vespertilionidae: *Corynorhinus*): inter- and intraspecific relationships inferred from mitochondrial and nuclear DNA sequences. Mol Phylogenet Evol 37:762–775

Pierson ED, Wackenhut MC, Altenbach JS et al (1999) Species conservation assessment and strategy for Townsend's big-eared bat (*Corynorhinus townsendii townsendii and Corynorhinus townsendii pallescens*). Idaho Conservation Effort, Idaho Department of Fish and Game, Boise, ID

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43:223–225

Ryder OA (1986) Species conservation and systematics: the dilemma of subspecies. Trends Ecol Evol 1:9–10

Schneider S, Roessli D, Excoffier L (2000) Arlequin ver. 2.000: a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland

Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. Can J Zool 69:82–90

Sherwin RE, Stricklan D, Rogers DS (2000a) Roosting affinities of Townsend's big-eared bat (*Corynorhinus townsendii*) in northern Utah. J Mammal 81:939–947

Sherwin RE, Gannon WL, Altenbach JS, Stricklan D (2000b) Roost fidelity of Townsend's big-eared bat in Utah and Nevada. Trans West Sect Wildl Soc 36:15–20

Sherwin RE, Gannon WL, Altenbach JS (2003) Managing complex systems simply: understanding inherent variation in the use of roosts by Townsend's big-eared bat. Wildl Soc Bull 3:62–72

Slatkin M (1993) Isolation by distance in equilibrium and non-equilibrium populations. Evolution 47:264–279

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457–462

Smith H, Chiszar D, Montanucci R (1997) Subspecies and classification. Herpetol Rev 28:13–16

Sokal RR, Rohlf FJ (1995) Biometry. Freeman, New York

Stihler CW, Jones A, Wallace JL (1997) Use of Elkhorn Cave, Grant County, West Virginia, by a bachelor colony of *Corynorhinus townsendii virginianus* (abstract). Bat Res News 38:130

Storz JF (2000) Variation at tri- and tetranucleotide repeat microsatellite loci in the fruit bat genus *Cynopterus* (Chiroptera: Pteropodidae). Mol Ecol 9:2199–2201

Swofford DL (2003) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts

Szewczak JM, Szewczak SM, Morrison ML, Hall LS (1998) Bats of the White and Inyo Mountains of California-Nevada. Great Basin Nat 58:66–75

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Tuttle MD (1977) Gating as a means of protecting cave dwelling bats. In: Ailey T, Rhodes D (eds) National Cave Management Symposium Proceedings. Speleobooks, Albuquerque, New Mexico pp 77–82

Tuttle MD, Taylor DAR (1994) Bats and mines. Resource Publication Bat Conser Int 3:1–41

United States Fish and Wildlife Service (1979) Endangered and threatened wildlife and plants; listing of the Virginia and Ozark Big-Eared Bats as endangered species, and critical habitat determination. Fed Regist 44:69206–69208

United States Fish and Wildlife Service (1989) Endangered and Threatened Wildlife and Plants; Animal Candidate Review for Listing as Endangered or Threatened Species. Fed Regist 54:54554–54579

United States Fish and Wildlife Service (1994) Endangered and Threatened Wildlife and Plants; Animal Candidate Review for Listing as Endangered or Threatened Species. Fed Regist 59: 58988

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535

Vonhof MJ, Davis CS, Fenton MB, Strobeck C (2002) Characterization of dinucleotide microsatellite loci in big brown bats (*Eptesicus fuscus*), and their use in other North American verspertilionid bats. Mol Ecol Notes 2:167–170

Weber JL, Wong C (1993) Mutation of human short tandem repeats. Hum Mol Genet 2:1123–1128

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. Evolution 38:1358–1370

Western Bat Working Group (1998) The western bat species: regional priority matrix. Western Bat Working Group. Reno, Nevada

Weyandt SE, van den Bussche R, Hamilton MJ, Leslie DM (2005) Unraveling the effects of sex and dispersal: conservation genetics of the endangered Ozark big-eared bat (*Corynorhinus townsendii ingens*). J Mammal 85:140–148

Wilmer JW, Barratt E (1996) A non-lethal method of tissue sampling for genetic studies of chiropterans. Bat Res News 37:1–4

Wilmer JW, Moritz C, Hall L, Toop J (1994) Extreme population structuring in the threatened ghost bat, *Macroderma gigas*: evidence from mitochondrial DNA. Proc R Soc Lond B Biol Sci 257:193–198

Wilmer JW, Hall L, Barratt E, Moritz C (1999) Genetic structure and male-mediated gene flow in the ghost bat (*Macroderma gigas*). Evolution 53:1582–1591

Yang Z, Goldman N, Friday A (1994) Comparison of models for nucleotide substitution used in maximum likelihood phylogenetic estimation. Mol Biol Evol 11:316–324